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Intranasal vaccination with H5, H7 and H9 hemagglutinins co-localized in a virus-like particle protects ferrets from multiple avian influenza viruses

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ABSTRACT

Avian influenza H5, H7 and H9 viruses top the World Health Organization's (WHO) list of subtypes with the greatest pandemic potential. Here we describe a recombinant virus-like particle (VLP) that co-localizes hemagglutinin (HA) proteins derived from H5N1, H7N2, and H9N2 viruses as an experimental vaccine against these viruses. A baculovirus vector was configured to co-express the H5, H7, and H9 genes from A/Viet Nam/1203/2004 (H5N1), A/New York/107/2003 (H7N2) and A/Hong Kong/33982/2009 (H9N2) viruses, respectively, as well as neuraminidase (NA) and matrix (M1) genes from A/Puerto Rico/8/1934 (H1N1) virus. Co-expression of these genes in Sf9 cells resulted in production of triple-subtype VLPs containing HA molecules derived from the three influenza viruses. The triple-subtype VLPs exhibited hemagglutination and neuraminidase activities and morphologically resembled influenza virions. Intranasal vaccination of ferrets with the VLPs resulted in induction of serum antibody responses and efficient protection against experimental challenges with H5N1, H7N2, and H9N2 viruses.

Introduction

Avian influenza represents an acute threat to public health (Morens and Fauci, 2012; Palese, 2006; Yen and Webster, 2009). With an increasing number of infections of highly pathogenic avian influenza (HPAI) of the H5N1 subtype, there is a concern that this virus could cause a pandemic (Kang et al., 2009; Morens and Fauci, 2012). In addition to H5N1, other potentially pandemic viruses of avian origin exist. The H7N7 and H7N2 viruses caused human infections in the past indicating the potential threat posed by H7 subtype viruses (Belser et al., 2008; Pappas et al., 2007). Avian H9N2 influenza was also identified as a human pathogen (Blanco et al., 2013; Pushko et al., 2005; Yen and Webster, 2009). Frequent genetic changes, multiple strains capable of reassortment, and the possibility of genetic manipulation represent challenges for influenza vaccine development (Morens and Fauci, 2012; Palese, 2004). Prediction of pandemic virus is a challenging task; therefore, a prepandemic vaccine that induces immunity to multiple potentially pandemic subtypes can be important for pandemic preparedness including vaccine stockpiling options (Rebmann and Zelicoff, 2012). This is emphasized by the outbreak of H7N9 virus in China. In the case of a pandemic, such prepandemic vaccines could decrease the severity of disease and save lives during a pandemic until a specific pandemic vaccine is made (Oxford et al., 2006; Palese, 2006). However, for financial reasons it is not feasible to make a multivalent prepandemic vaccine using current commercial technology. Classic trivalent inactivated vaccines represent mixtures of inactivated H1N1, H3N2, and influenza B viruses (Kang et al., 2009; Palese, 2006; Pushko, 2009). Each virus is grown separately in fertilized eggs, inactivated, and then combined with two other strains to make a trivalent vaccine. Live attenuated influenza vaccines are also initially prepared in eggs and then blended to produce a trivalent vaccine (Hussain et al., 2010). Preparation of pandemic vaccines is costly and the need for individual preparation of vaccines for multiple strains increases the cost of a trivalent vaccine. The use of eggs in vaccine production is another weakness. Although the first cell-based and recombinant subunit influenza vaccines have been introduced (Plosker, 2012; Treanor et al., 2011) and recently approved by the regulatory agencies, the majority of influenza vaccines are still made in eggs and in the event of an outbreak of avian influenza or other disease that affects chicken flocks, the supply of vaccine could be threatened.

Recombinant virus-like particles (VLPs) have been recently shown as a promising vaccine approach for influenza (Galarza et al., 2005; Kang et al., 2009; Perrone et al., 2009; Pushko et al.; Pushko et al., 2005; Quan et al.; Ross et al., 2009). Recombinant VLPs are produced by using cell culture methods and do not require egg-based technology for the production. In a recent study, we described novel

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multi-subtype VLPs containing three subtypes of HA within the envelope (Pushko et al., 2011). By immunoelectron microscopy, we have shown that multiple subtypes of HA can co-localize within VLP and that such multi-subtype VLPs induce highly protective immune responses against multiple strains of influenza when administered intramuscularly (i.m.) (Pushko et al., 2011). In the current study, we configured a triple-subtype VLP to contain the H5, H7, and H9 proteins derived from three potentially pandemic influenza viruses and evaluated immunogenicity and efficacy of such H5/H7/H9 VLPs following intranasal (i.n.) vaccination. We demonstrate that i.n. vaccination with the H5/H7/H9 triple-subtype VLP induced immune responses and protected ferrets from experimental challenges with three avian influenza viruses. Potential advantages of multi-subtype VLPs for pandemic preparedness strategies are discussed.

Results

Preparation of triple-subtype H5/H7/H9 VLPs

For preparation of triple-subtype VLPs, the HA proteins were derived from three avian-origin influenza viruses, H5N1, H7N2 and H9N2. The HPAI H5N1 virus, VN/04 of clade 1, was isolated from a fatal human case and caused 100% lethality in ferrets (Maines et al., 2005). The H7N2 virus, NY/03, was isolated from a hospitalized patient that recovered and subsequently found to replicate efficiently in the upper respiratory tract of ferrets (Belser et al., 2008; Ostrowsky et al., 2012). The H9N2 virus, HK/09 of G1 clade, was originally isolated from a nasopharyngeal aspirate of an adult female patient (Cheng, 2010).

In order to prepare triple-subtype H5/H7/H9 VLP vaccine, the rBV vector was made that co-expressed H5, H7, H9, as well as NA and M1 genes (Fig. 1a). The H5, H7 and H9 genes encoded polypeptides of 568 aa, 552 aa, and 560 aa in length with predicted average molecular masses of 64.5 kilodalton (kDa), 61.5 kDa and 62.9 kDa, respectively. The NA and M1 were 454 aa and 252 aa in length and had expected molecular masses of 50.1 kDa and 27.9 kDa, respectively. Both NA and M1 have been previously shown to be important structural constituents of influenza VLPs (Pushko et al., 2011, 2005) and therefore were included in the expression construct. The NA and M1 genes were derived from PR8 virus, which is standard strain used in commercial reassortant vaccines. For preparation of VLPs, Sf9 cells were infected with rBV at an MOI of 3 to allow expression of H5, H7, H9, NA, and M1 genes, and the VLPs were harvested from culture supernatant.

Characterization of triple-subtype VLPs

Triple-subtype H5/H7/H9 VLPs were concentrated 100-fold and partially purified by ultracentrifugation. The presence of H5, H7, and H9 proteins in VLPs was confirmed by SDS-PAGE and western blot (Fig. 1b), as well as by IFA in Sf9 cells infected with recombinant BV (Fig. 1c). The HAs within VLPs represented uncleaved HA0 polypeptides of approximately 62–64 kDa, while the NA was not detected by western blot suggesting low level of cross-reactivity with the H5N1-, H7N2-, and H9N2-specific antisera used in western blot. A band of approximately 25 kDa corresponding to M1 protein was detected by western blot with H9N2-specific antiserum, but not with H5N1 or H7N2 antisera. By gel staining, a band of approximately 40 kDa was observed, which was not detectable by antisera in western blot and could represent an rBV- or Sf9 cell-derived contaminant.

The ability of H5/H7/H9 VLPs to agglutinate turkey RBCs was confirmed by hemagglutination assay (Fig. 2a). For hemagglutination assay, dilutions of VLPs were mixed with turkey RBCs recommended by the WHO as a reagent for animal influenza diagnosis and surveillance (WHO, 2012b). The complete hemagglutination activity at the titer of 1:2048 of unconcentrated VLPs confirmed that the incorporated HA proteins retained their stability and binding activities (Fig. 2a). The size and morphology of triple-subtype H5/H7/H9 VLPs were further examined by negative-staining transmission electron microscopy. The H5/H7/H9 VLPs were identified as largely spherical, typical influenza-like enveloped particles approximately 90–120 nm in diameter and characteristic prominent spikes protruding from the VLP envelope (Fig. 2b).

The functional NA enzymatic activity of VLPs was confirmed by using standard fluorescence-based influenza neuraminidase assay (Fig. 2c).

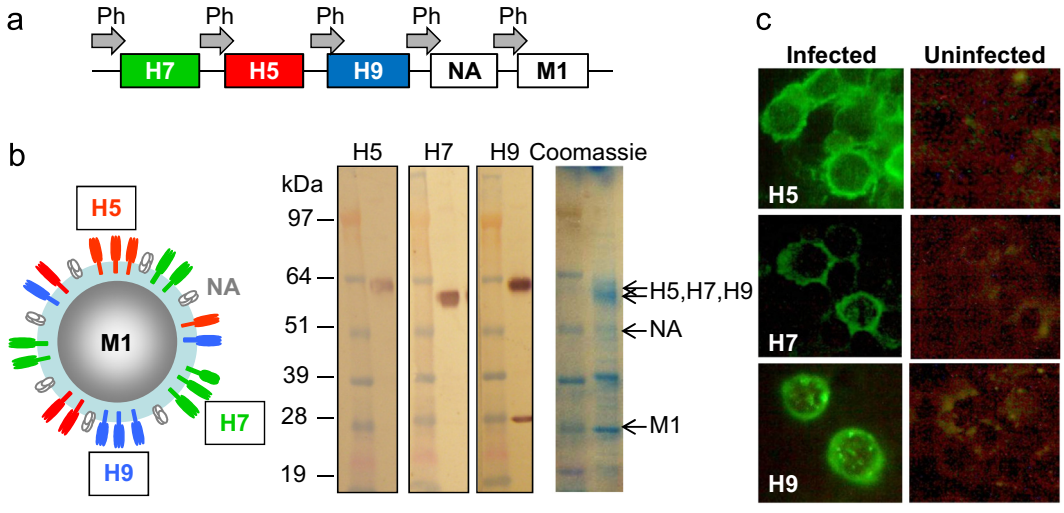

Fig. 1. Preparation of triple-subtype VLPs that co-localize HA proteins from H5, H7 and H9 subtype viruses. (a) Recombinant baculovirus (rBV) for expression of triple-subtype VLPs in *Spodoptera frugiperda* (Sf9) cells. Influenza HA gene sequences were derived from VN/04 (H5N1), NY/03 (H7N2), and HK/09 (H9N2) viruses. NA and M1 genes were from PR8 virus (see text for virus abbreviations). The H5, H7, H9, NA, and M1 genes were combined within recombinant rBV in a tandem fashion so that each gene was expressed from its own polyhedrin (Ph) promoter. (b) Schematic depiction of triple-subtype VLPs and detection of HA proteins within VLPs by SDS-PAGE and western blot. Locations of influenza proteins and SeeBlue Plus2 molecular weight standards are shown. Western blot was done using ferret antisera against VN/04, NY/03, and HK/09 viruses followed by alkaline phosphatase-conjugated anti-ferret goat IgG (H+L). (c) Immunofluorescence assay (IFA) of Sf9 cells infected with recombinant baculovirus expressing multiple HA subtypes (left) or uninfected control Sf9 cells (right). Indicated subtype-specific ferret antisera were used for evaluating expression of respective antigens in infected and uninfected control Sf9 cells.

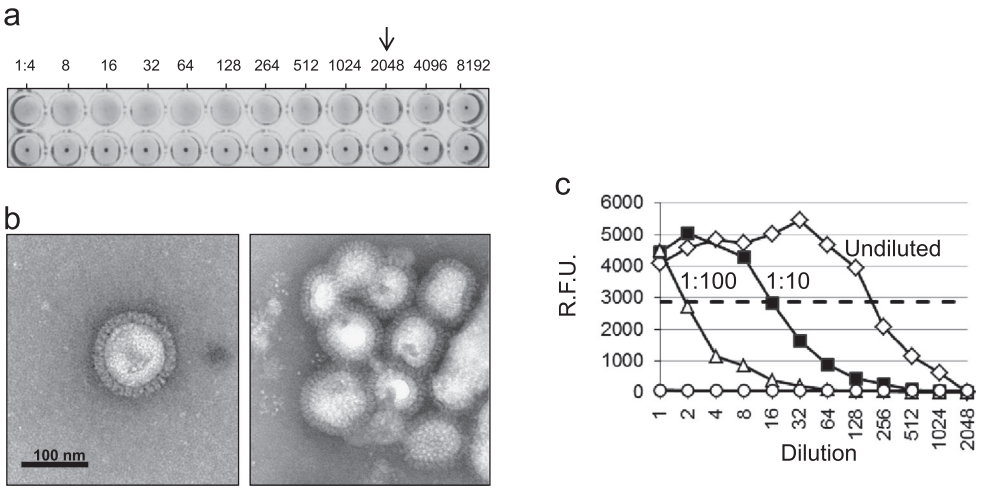

Fig. 2. Characterization of triple-subtype VLPs, by (a) hemagglutination assay, (b) negative staining transmission electron microscopy and (c) neuraminidase enzyme assay. (a) VLPs were harvested from the culture medium of rBV-infected Sf9 cells on day 3. VLPs were concentrated 100-fold, partially purified by 20% step sucrose gradient ultracentrifugation, and resuspended in PBS buffer. Hemagglutination test was done on unconcentrated VLPs using turkey erythrocytes. Arrow indicates the HA titer. (b) For transmission electron microscopy, concentrated VLPs were stained with 1% phosphotungstic acid. Bar, 100 nm. (c) For neuraminidase fluorescence-based assay, VLPs were initially diluted 10-fold (filled squares) or 100-fold (open triangles), or tested undiluted (open diamonds). Negative control (PBS) is indicated with open circles. Normalization line is also shown (dashed). The NA enzymatic activity was measured in Relative Fluorescent Units (R.F.U.).

Immunogenicity and protective efficacy of H5/H7/H9 triple-subtype VLPs in ferrets after intranasal vaccination

Ferrets represent highly relevant animal model for influenza (Cheng et al., 2009; Huang et al., 2011; Perrone et al., 2009). Ferrets received two vaccinations i.n. of the H5/H7/H9 triple-subtype VLPs and control animals received PBS in place of vaccine. The i.n. route was chosen because we previously showed that i.m. vaccination with triple-subtype VLPs resulted in protective responses (Pushko et al., 2011). There were no adverse effects from the vaccine in ferrets and similar weight gains were observed between vaccinated and PBS control animals (not shown). The hemagglutination inhibition (HI) and virus neutralizing (VN) antibody responses to each homologous virus were measured in individual serum samples collected prior to vaccine boost and challenge. After the first vaccine dose, HI antibody was elicited to H7 (titer of 10) and H9 (titer range 20 to 80) antigens, whereas no HI antibody was detected to H5 antigen. This is in line with prior observations of low immunogenicity of H5 protein (Treanor et al., 2006). As expected, no HI antibody was detected in control animals. After boost vaccination, both HI and VN titers to all three homologous viruses were detected in animals vaccinated with triple-subtype VLPs, but not in control animals (Table 1). In the sera from ferrets vaccinated with VLPs, the highest HI and VN titers were observed to HK/09 virus, while the lowest antibody titers were to VN/04 virus (Table 1). No HI antibody was detected to heterologous H5N1 viruses A/Egypt/4935/09 (clade 2.2.1) and A/Duck/Vietnam/1206/12 (clade 2.3.2.1) (data now shown).

Vaccine protection was measured by survival, reduction in fever, weight loss, and viral shedding in ferrets following homologous virus challenges. Although 10^6 EID_{50} challenges with H7N2 or H9N2 viruses did not result in a lethal outcome, PBS control ferrets challenged with H5N1 virus, developed severe disease and had to be euthanized on days 5–6 p.c. due to severe neurological symptoms. In spite of the lowest pre-challenge HI and VN antibody titers to H5N1, all VLP-vaccinated animals survived challenge with H5N1 virus (Table 1). H7N2 and H5N1 challenge viruses induced an increase in body temperature among control ferrets with mean maximum fever of 1.1 °C and 2.0 °C over baseline, respectively (Table 1). The rise in body temperature among VLP vaccinated ferrets after H7N2 and H5N1 challenge was less than that of controls, with mean maximum fever of 0.3 °C and 0.6 °C over baseline, respectively. In spite of the strong H9N2 HA and VN response (Table 1), the difference in body temperatures of control and vaccinated ferrets after H9N2 virus challenge was less apparent and statistically not significant. This was consistent with the previous study, in which only part of H9N2-infected ferrets showed transient elevation of temperature (Wan et al., 2008).

The triple-subtype VLPs also provided protection against weight loss in H5N1 and H9N2 challenged groups. In particular, on day 6 p.c., VLP-vaccinated ferrets lost 4.7% of starting weight after challenge with H5N1 virus, compared with 13.4% weight loss for the control ferrets (Table 1). Following challenge with H9N2 virus, vaccinated ferrets lost 1.8% of weight, whereas control animals lost 6.9% of their pre-challenge weight. The difference in body weights of control and vaccinated ferrets after H7N2 virus challenge was less apparent; some control animals have increased weight after challenge, however the differences were not significant.

The extent of virus replication in the upper respiratory tract was determined by titrating nasal wash samples collected from immune and control ferrets following challenge with homologous virus. All three subtypes replicated efficiently in the upper respiratory tract of unimmunized control ferrets for six days p.c. (Fig. 3). In comparison, immunized animals displayed a significant reduction in H5N1, H7N2 and H9N2 viral load. Moreover, the majority of VLP-immune ferrets cleared H5N1 and H7N2 virus two days earlier than control ferrets. Taken together, these data demonstrate that H5/H7/H9 triple-subtype VLP immunization via i.n. route offered significant protection against substantial fever, weight loss, and viral shedding following homologous virus challenge, as well as against severe neurological disease in case of H5N1 virus infection.

Discussion

Annual influenza epidemics affect millions of people worldwide causing incapacitating illnesses that can result in hospitalization or death, especially in the elderly, the very young and those with underlying health conditions. Pandemic influenza represents even a greater threat than seasonal epidemics. The “Spanish” influenza of 1918 caused by H1N1 subtype virus was the most devastating pandemic in the human history and responsible for

Table 1
Antibody responses and clinical signs of infection following challenge with A/Vietnam/1203/04 (H5N1), A/New York//107/03 (H7N2), or A/Hong Kong/33982/09 (H9N2) viruses.

Virus	Vaccine status	HI titer range (GMT)[a]	VN titer range (GMT)	Mean max temperature increase (°C)	% Mean weight loss [b] (*t* test)	Mean peak virus titer (log10 EID or PFU/ml)	Survival
VN/04H5N1	VAX	5–10 (7.1)	10–20 (13.3)	0.6	4.7 (0.08)	3.9	4/4
	PBS	< 10	< 10	2.0	13.4	5.6	0/4[c]
NY/03H7N2	VAX	10–40 (23.8)	10–160 (35.6)	0.3	4.7 (0.47)	4.8	4/4
	PBS	< 10	< 10	1.1	2.3	6.1	4/4
HK/09H9N2	VAX	20–80 (33.6)	20–160 (63.5)	1.2	1.8 (0.01)	4.0	4/4
	PBS	< 10	< 10	0.7	6.9	6.2	4/4

[a] As measured in pre-challenge sera, turkey RBCs were used to assess antibody response to NY/03 and HK/09 viruses, horse RBCs were used to assess antibody response to VN/04 virus.
[b] Measured at day 6 p.c.
[c] Two ferrets were euthanized on day 5 and two were euthanized on day 6 post challenge due to neurological symptoms.

the deaths of 40 to 100 million people globally (Pushko, 2009; Taubenberger et al., 2012). Multiple potentially pandemic viruses continue to circulate in birds and other animals. In particular, avian influenza viruses of H5, H7, and H9 subtypes have been identified as pathogens of concern and potentially pandemic viruses (Belser et al., 2008; Palese, 2004; Pappas et al., 2007; WHO, 2013). In addition to the potential of causing lethal human disease and pandemics, avian influenza can cause devastating epizootics in poultry that can threaten food supply and safety, and also result in reduced capability to manufacture egg-dependent vaccines worldwide including those against influenza, yellow fever, mumps and measles viruses. Therefore, vaccines capable of protecting against multiple potentially pandemic influenza strains are needed. Candidate vaccines against H5, H7,and H9 viruses have been prepared in the past (Couch et al., 2012). In order to enhance pandemic preparedness, inactivated H5N1 vaccines have been developed and approved by regulatory agencies (O'Neill and Donis, 2009) including recently approved cell culture-derived Flucelvax vaccine. Both the VN/04 (H5N1) and HK/09 (H9N2) viruses are on the list of candidate vaccines recommended by the World Health Organization (WHO) in 2012 for pandemic preparedness (WHO, 2012a). However, although H7 and H9 candidate vaccine strains exist, there are currently no approved vaccines available for human use for these subtypes (WHO, 2012a).

Recombinant multi-HA VLPs described in this study represent a conceptually novel multi-specific influenza vaccine, which is designed to elicit specific immunity to H5, H7, and H9 influenza strains and does not require blending of individual vaccines to prepare a trivalent formulation. Previously, we have shown by immunoelectron microscopy that multi-subtype VLP co-localize multiple subtypes of hemagglutinin within the same particle, thus acquiring capability to protect against multiple influenza viruses following i.m. vaccination (Pushko et al., 2011). In the current study, we showed that H5/H7/H9 triple-subtype VLPs combine neutralizing epitopes from three influenza subtypes and protect from three potentially pandemic viruses even when administered mucosally.

Alternative approaches for preparation of broadly protective influenza vaccine include blended trivalent vaccine formulations as well as the "universal" vaccine approaches involving consensus sequences of HA protein or highly conserved influenza epitopes, for example the ectodomain of M2 ion channel protein (Denis et al., 2008; Ebrahimi et al.; Schotsaert et al., 2009). Universal vaccine approaches also include induction of immune response to conserved regions of HA (Pica and Palese, 2013; Rao et al., 2010; Wang and Palese, 2009; Wei et al., 2010). Our results suggest that in addition to blended trivalent and universal vaccine approaches, the multi-subtype VLPs can also induce immune responses and protection against multiple influenza viruses following i.m. and i.n. vaccinations. Similarly to the previously reported i.m. vaccination (Pushko et al., 2011), the protection observed in the current study in mucosally vaccinated ferrets is likely due to the production of virus-neutralizing antibodies directed against the HA. Although neutralizing antibody levels to H5N1 virus were low (10–20), mucosally vaccinated animals were protected against death following a lethal H5N1 virus challenge. Other immune responses not measured in the present study, in particular mucosal and T-cell-mediated immunity, may have contributed to protection (Nguyen et al., 1999; Pillet et al., 2011; Tumpey et al., 2001). Assessments in vivo of mucosal immune functions following influenza virus infection have been limited owing to a lack of ferret-specific reagents and incomplete genome sequencing of this species. Furthermore, the present proof-of-concept study was focused on detection of shedding virus in the nasal secretions and not designed for reliable evaluation of mucosal IgG and IgA. Detailed evaluation of mucosal IgG and IgA responses including heterosubtypic immunity in the i.n. vaccinated animals will be addressed in a separate study.

Since this is a novel approach, additional research and preclinical testing is needed. It is not known at this time if distinct HA subtypes form homo- or heterotrimers on the surface of VLPs. Previous research suggested that mixed trimers are not formed between the HAs of distinct subtypes and that incorrectly folded HA molecules are excluded during intracellular transport and exocytosis (Boulay et al., 1988; Chao, 1992). Furthermore, future studies will include development of quantitation methods for each HA subtype within triple-subtype VLPs. For this purpose, the single radial immunodiffusion (SRID) test recommended by the Center for Biologics Evaluation and Research (CBER) can be employed. These studies are hampered by unavailability of reagents to H7 and H9 subtypes and will be accomplished when reference standards become available. Previous electron microscopy and SRID studies on triple-subtype VLPs containing seasonal influenza subtypes have shown that distinct HA subtypes are co-localizing within multi-subtype VLPs at approximately equivalent quantities (Pushko et al., 2011). In case if certain HA subtypes require higher quantities for optimal immunogenicity, their presence within multi-subtype VLPs can potentially be improved by genetic engineering. For example, incorporation of HIV *env* protein into VLPs could be improved more than 10-fold by the rational design of leader and transmembrane domains of *env* (Wang et al., 2007). Additional data are also needed regarding protective effects of triple-subtype VLPs against heterologous challenges. It has been previously shown that mucosally vaccinated ferrets have broader protection against challenge with unrelated strains than parenterally vaccinated animals (Perrone et al., 2009). In summary, the current study shows that triple-subtype H5/H7/H9 VLPs can be a promising option for prepandemic vaccine that can be stockpiled and in the case of a pandemic involving either H5, H7 or H9 avian influenza viruses, could be used as a first line defense during the

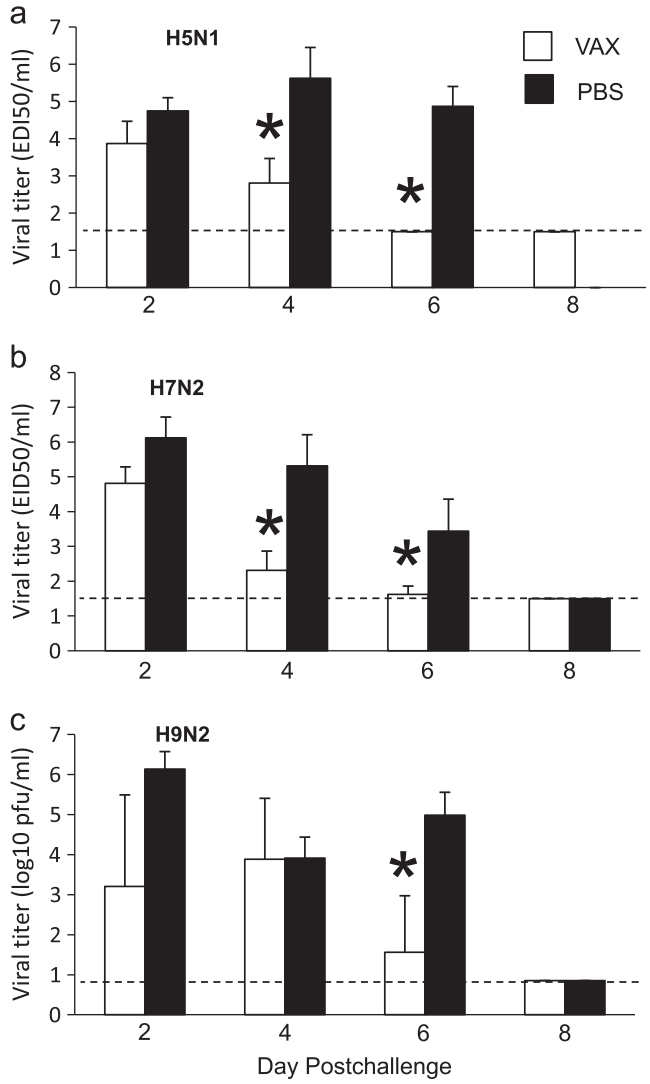

Fig. 3. Virus titers in the nasal cavities of VN/04 or NY/03 or HK/09—challenged ferrets previously inoculated with triple-subtype VLPs or PBS. Twelve ferrets were vaccinated intranasally (i.n.) with 15 μg (based on HA content) of triple-subtype VLPs two times, five weeks apart and randomly assigned to groups for virus challenge. Control ferrets were inoculated with PBS on the same schedule as vaccinated ferrets. Vaccinated and PBS-control ferrets were challenged with 10^6 EID50 VN/04 (H5N1) virus or 10^6 EID50 NY/03 (H7N2) virus or 10^6 PFU HK/09 (H9N2) virus. Nasal cavities of all ferrets were washed with 1ml PBS every other day starting two days p.c. and titrated in either eggs or standard plaque assay; averages of all four ferrets in each group are reported, error bars represent standard deviation. Open bars represent vaccinated ferrets, filled bars represent PBS-control ferrets, dotted lines indicate limit of detection for titration in eggs (top and middle panel) or plaque assay (bottom panel). Significant differences in titers between vaccinated and PBS-control groups, as analyzed by Student's *t*-test, are marked with an asterisk ($^*p \leq 0.045$).

time period that is required to make a vaccine against a specific pandemic virus. If proven safe and efficacious, this generic approach can also be applicable to other influenza viruses including seasonal strains.

Materials and methods

Viruses, plasmids and cells

Influenza HA gene sequences were derived from A/Viet Nam/1203/2004 (H5N1), A/New York/107/2003 (H7N2) and A/Hong Kong/33982/2009 (H9N2) viruses, further designated VN/04, NY/03, and HK/09, respectively. The sequences for HA genes were from the GenBank accession no. ABP51977.1, ACC55270.1 and ADC41843.1, respectively. All three HA genes were codon-optimized and synthesized (Genscript, Piscataway, NJ) for high-level expression in *Spodoptera frugiperda* (Sf9) cells (ATCC, Manassas, VA). The NA and M1 gene sequences, GenBank CY105898.1 and V01099.1, respectively, were derived from A/Puerto Rico/8/1934 (H1N1) virus, further designated PR8. In order to generate triple-subtype pandemic VLPs, three full-length HA genes, as well as NA and M1 genes were cloned within a single baculovirus vector, each gene within its own transcriptional cassette that included a polyhedrin promoter upstream from each gene. Recombinant baculovirus (rBV) expressing H5, H7, H9, NA, and M1 genes was generated using a Bac-to-Bac baculovirus expression system (Life Technologies, Carlsbad, CA). The titers of rBV preparations were determined by standard plaque assay in Sf9 cells.

Preparation of triple-subtype H5/H7/H9 VLPs

Sf9 cells were maintained as suspension cultures in SF900II-SFM insect serum free medium (Life Technologies, Carlsbad, CA) at 27 °C. For production of H5/H7/H9 VLPs, Sf9 cells were adjusted to 2×10^6 cells/ml and infected at a multiplicity of infection (MOI) of 3.0 for 72 h with rBV expressing H5, H7, H9, NA and M1 genes. Immunofluorescense assay (IFA) was done using ferret antisera specific for H5, H7, and H9 influenza (CDC, Atlanta, GA). For fluorescent staining, 0.3 ml aliquots of infected Sf9 cells were seeded into eight-well Nunc LabTek slides. Following 72 h incubation at 27 °C, Sf9 cells were fixed with cold acetone, and IFA was carried out as described elsewhere (Pushko et al., 2001). Antigen-expressing cells were visualized using FITC-conjugated goat anti-ferret IgG (H+L) (KPL, Gaithersburg, MD). VLPs were harvested from the growth medium supernatant, concentrated and partially purified by using a 20% (w/v) sucrose step gradient in phosphate buffered saline (PBS). VLPs were stored at 2–8 °C in a PBS buffer until vaccinations. The SDS-PAGE was done in 4–12% polyacrylamide gels (Life Technologies, Carlsbad, CA) followed by staining with GelCode Blue stain (Pierce, Rockford, IL). Western blots were carried out by using ferret subtype-specific antisera followed by alkaline phosphatase-conjugated goat anti-ferret IgG (H+L). The HA content was estimated by determining total protein concentration using fluorometric method (Qubit 2.0, Life Technologies) followed by densitometry analysis of stained gel and calculating total HA protein content from the percentage of the HA bands.

Hemagglutination and neuraminidase assays

For hemagglutination assay, VLPs were serially diluted at 2-fold increments in 50 μl volume in a 96-well plate. To each VLP dilution, 50 μl of 0.5% turkey red blood cell (RBC) working solution was added, mixtures of VLPs and RBCs were gently agitated and the plate was incubated at room temperature for 30–60 min before examination. Negative hemagglutination results appeared as dots in the center of the wells. The titer was calculated as the highest dilution factor that produced a positive reading. The functional neuraminidase enzymatic activity was determined by using a fluorescence-based NA assay (NA-Fluor, Life Technologies) with methyl umbelliferone *N*-acetyl neuraminic acid (MUNANA; Sigma, St Louis, MO) as a substrate according to manufacturer's instructions. Diluent (saline) was used as a negative control.

Electron microscopy of H5/H7/H9 VLPs

VLP samples were adsorbed onto a freshly discharged 400 mesh carbon parlodion-coated copper grids (Poly-Sciences, Warrington, PA). The grids were rinsed with buffer containing 20 mM Tris,

pH 7.4, and 120 mM KCl and negatively stained with 1% phosphotungstic acid, then dried by aspiration. VLPs were visualized on a Hitachi H-7600 transmission electron microscope (Hitachi High Technologies America, Schaumburg, IL) operating at 80 kV and digitally captured with a CCD camera at 1 k × 1 k resolution (Advanced Microscopy Techniques Corp., Danvers, MA).

Serological assays.

All sera were initially diluted 1:10 in receptor-destroying enzyme from Vibrio cholerae (Denka Seiken, Tokyo, Japan). Hemagglutination inhibition (HI) assay was performed using 0.5% turkey or 1% horse erythrocytes with 4 HA units of homologous viruses using standard methods (Pushko et al., 2005). Horse RBCs were used to assess HI antibody response to VN/04 virus. Titers of virus neutralizing (VN) antibody were determined essentially as described (Mozdzanowska et al., 1997) and expressed as the reciprocal of the highest dilution of serum that neutralized 100–200 plaque forming units of virus in MDCK cell cultures. The neutralization titers were also presented as the geometric mean titers (GMT) from vaccinated or control ferrets.

Viruses for ferret challenge.

The virus stocks of VN/04 and NY/03 were grown in the allantoic cavities of ten-day-old embryonated hens' eggs at 37 °C for 24–26 or 40 h, respectively. The virus stock of HK/09 was grown in Madin–Darby Canine Kidney (MDCK) cells as previously described (Zeng et al., 2007). Pooled allantoic fluid or cell supernatant was clarified by centrifugation and aliquots were stored at −70 °C until use. A 50% egg infectious dose (EID50/ml) and plaque forming unit (PFU) titers in MDCK cells were determined using standard assay methods as previously described (Zeng et al., 2007). All experiments were performed in negative pressure biosafety level 3 laboratories with enhancements as outlined in the Biomedical Microbiological and Biomedical Laboratory.

Ferret vaccination and viral challenge

Adult male Fitch ferrets, 4 to 5 months of age (Triple F Farms, Sayre, PA), serologically negative by HI assay for currently circulating influenza viruses, were used in this study. For vaccinations, ferrets were each inoculated 2 times (5 weeks between inoculations) intranasally (i.n.) with 15 μg of total HA in the VLPs (vaccinated) or with PBS (controls). The vaccine dose of 15 μg and route of administration were consistent with the previous human clinical trial of purified HA antigen (Stephenson et al., 2006). Prior to primary vaccination, vaccine boost, and viral challenge, all ferrets were bled for collection of serum to assess response to vaccination and randomly assigned for challenge with VN/04 virus, NY/03 virus, or HK/09 virus (4 vaccinated and 4 PBS controls for each challenge group). Following anesthesia with an intramuscular injection of a ketamine–xylazine–atropine cocktail, ferrets were challenged i.n. with 10^6 EID50 (VN/04 and NY/03) or 10^6 PFU (HK/09) of virus in a total volume of 1 ml (500 μl per nostril) diluted in PBS. Viral challenge occurred 5.5 to 7.5 weeks after vaccine boost. Following challenge, ferrets were monitored daily for changes in body weight and temperature, as well as clinical signs of illness. Nasal wash samples were collected at 2, 4, 6, and 8 days post-challenge (p.c.) and titrated in eggs (VN/04 and NY/03) or in a standard plaque assay (HK/09) to determine viral titers in the upper respiratory tract. The statistical significance of differences in weight loss, temperature changes, and virus titers between vaccinated and PBS-control animals were determined by Student's *t* test.

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References

Belser, J.A., Blixt, O., Chen, L.M., Pappas, C., Maines, T.R., Van Hoeven, N., Donis, R., Busch, J., McBride, R., Paulson, J.C., Katz, J.M., Tumpey, T.M., 2008. Contemporary North American influenza H7 viruses possess human receptor specificity: implications for virus transmissibility. Proc. Nat. Acad. Sci. U.S.A. 105, 7558–7563.

Blanco, J.C., Pletneva, L.M., Wan, H., Araya, Y., Angel, M., Oue, R.O., Sutton, T.C., Perez, D.R., 2013. Receptor characterization and susceptibility of cotton rats to avian and 2009 pandemic influenza virus strains. J. Virol. 87, 2036–2045.

Boulay, F., Doms, R.W., Webster, R.G., Helenius, A., 1988. Posttranslational oligomerization and cooperative acid activation of mixed influenza hemagglutinin trimers. J. Cell Biol. 106, 629–639.

Chao, C.C., 1992. A single amino acid deletion at the amino terminus of influenza virus hemagglutinin causes malfolding and blocks exocytosis of the molecule in mammalian cells. J. Biol. Chem. 267, 2142–2148.

Cheng, P.K.C.a.L., W.L. 2010. Molecular characterization of H9N2 isolated in Hong Kong from 2008 to 2009, Unpublished, Hong Kong, GenBank Acc. No. CY055137.

Cheng, X., Eisenbraun, M., Xu, Q., Zhou, H., Kulkarni, D., Subbarao, K., Kemble, G., Jin, H., 2009. H5N1 vaccine-specific B cell responses in ferrets primed with live attenuated seasonal influenza vaccines. PLoS One 4, e4436.

Couch, R.B., Decker, W.K., Utama, B., Atmar, R.L., Nino, D., Feng, J.Q., Halpert, M.M., Air, G.M., 2012. Evaluations for in vitro correlates of immunogenicity of inactivated influenza a h5, h7 and h9 vaccines in humans. PLoS One 7, e50830.

Denis, J., Acosta-Ramirez, E., Zhao, Y., Hamelin, M.E., Koukavica, I., Baz, M., Abed, Y., Savard, C., Pare, C., Lopez Macias, C., Boivin, G., Leclerc, D., 2008. Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. Vaccine 26, 3395–3403.

Ebrahimi, S.M., Tebianian, M., Aghaiypour, K., Nili, H., Mirjalili, A., Prokaryotic expression and characterization of avian influenza A virus M2 gene as a candidate for universal recombinant vaccine against influenza A subtypes; specially H5N1 and H9N2. Mol. Biol. Rep. 37, 2909–2914.

Galarza, J.M., Latham, T., Cupo, A., 2005. Virus-like particle (VLP) vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol. 18, 244–251.

Huang, S.S., Banner, D., Fang, Y., Ng, D.C., Kanagasabai, T., Kelvin, D.J., Kelvin, A.A., 2011. Comparative analyses of pandemic H1N1 and seasonal H1N1, H3N2, and influenza B infections depict distinct clinical pictures in ferrets. PLoS One 6, e27512.

Hussain, A.I., Cordeiro, M., Sevilla, E., Liu, J., 2010. Comparison of egg and high yielding MDCK cell-derived live attenuated influenza virus for commercial production of trivalent influenza vaccine: in vitro cell susceptibility and influenza virus replication kinetics in permissive and semi-permissive cells. Vaccine 28, 3848–3855.

Kang, S.M., Pushko, P., Bright, R.A., Smith, G., Compans, R.W., 2009. Influenza virus-like particles as pandemic vaccines. Curr. Top. Microbiol. Immunol. 333, 269–289.

Maines, T.R., Lu, X.H., Erb, S.M., Edwards, L., Guarner, J., Greer, P.W., Nguyen, D.C., Szretter, K.J., Chen, L.M., Thawatsupha, P., Chittaganpitch, M., Waicharoen, S., Nguyen, D.T., Nguyen, T., Nguyen, H.H., Kim, J.H., Hoang, L.T., Kang, C., Phuong, L. S., Lim, W., Zaki, S., Donis, R.O., Cox, N.J., Katz, J.M., Tumpey, T.M., 2005. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J. Virol. 79, 11788–11800.

Morens, D.M., Fauci, A.S., 2012. Emerging infectious diseases in 2012: 20 years after the institute of medicine report. mBio 3.

Mozdzanowska, K., Furchner, M., Washko, G., Mozdzanowski, J., Gerhard, W., 1997. A pulmonary influenza virus infection in SCID mice can be cured by treatment with hemagglutinin-specific antibodies that display very low virus-neutralizing activity in vitro. J. Virol. 71, 4347–4355.

Nguyen, H.H., Moldoveanu, Z., Novak, M.J., van Ginkel, F.W., Ban, E., Kiyono, H., McGhee, J.R., Mestecky, J., 1999. Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific CD8(+) cytotoxic T lymphocyte responses induced in mucosa-associated tissues. Virology 254, 50–60.

O'Neill, E., Donis, R.O., 2009. Generation and characterization of candidate vaccine viruses for prepandemic influenza vaccines. Curr. Top. Microbiol. Immunol. 333, 83–108.

Ostrowsky, B., Huang, A., Terry, W., Anton, D., Brunagel, B., Traynor, L., Abid, S., Johnson, G., Kacica, M., Katz, J., Edwards, L., Lindstrom, S., Klimov, A., Uyeki, T.M., 2012. Low pathogenic avian influenza A (H7N2) virus infection in immunocompromised adult, New York, USA, 2003. Emerg. Infect. Dis. 18, 1128–1131.

Oxford, J.S., Lambkin, R., Elliot, A., Daniels, R., Sefton, A., Gill, D., 2006. Scientific lessons from the first influenza pandemic of the 20th century. Vaccine 24, 6742–6746.

Palese, P., 2004. Influenza: old and new threats. Nat. Med. 10, S82–87.

Palese, P., 2006. Making better influenza virus vaccines? Emerg. Infect. Dis. 12, 61–65.
Pappas, C., Matsuoka, Y., Swayne, D.E., Donis, R.O., 2007. Development and evaluation of an Influenza virus subtype H7N2 vaccine candidate for pandemic preparedness. Clin. Vaccine Immunol. 14, 1425–1432.
Perrone, L.A., Ahmad, A., Veguilla, V., Lu, X., Smith, G., Katz, J.M., Pushko, P., Tumpey, T.M., 2009. Intranasal vaccination with 1918 influenza virus-like particles protects mice and ferrets from lethal 1918 and H5N1 influenza virus challenge. J. Virol. 83, 5726–5734.
Pica, N., Palese, P., 2013. Toward a universal influenza virus vaccine: prospects and challenges. Annu. Rev. Med. 64, 189–202.
Pillet, S., Kobasa, D., Meunier, I., Gray, M., Laddy, D., Weiner, D.B., von Messling, V., Kobinger, G.P., 2011. Cellular immune response in the presence of protective antibody levels correlates with protection against 1918 influenza in ferrets. Vaccine 29, 6793–6801.
Plosker, G.L., 2012. A/H5N1 prepandemic influenza vaccine (whole virion, vero cell-derived, inactivated) [Vepacel(R)]. Drugs 72, 1543–1557.
Pushko, P., Geisbert, J., Parker, M., Jahrling, P., Smith, J., 2001. Individual and bivalent vaccines based on alphavirus replicons protect guinea pigs against infection with Lassa and Ebola viruses. J. Virol. 75, 11677–11685.
Pushko, P., Kort, T., Nathan, M., Pearce, M.B., Smith, G., Tumpey, T.M., Recombinant H1N1 virus-like particle vaccine elicits protective immunity in ferrets against the 2009 pandemic H1N1 influenza virus. Vaccine 28, 4771–4776.
Pushko, P., Pearce, M.B., Ahmad, A., Tretyakova, I., Smith, G., Belser, J.A., Tumpey, T. M., 2011. Influenza virus-like particle can accommodate multiple subtypes of hemagglutinin and protect from multiple influenza types and subtypes. Vaccine 29, 5911–5918.
Pushko, P,R.B, Tumpey, T., Smith, G., 2009. Engineering better influenza vaccines: traditional and new approaches. In: Khudyakov, YE (Ed.), Medicinal Protein Engineering, 2009. CRC Press, Boca Raton, pp. 169–204.
Pushko, P., Tumpey, T.M., Bu, F., Knell, J., Robinson, R., Smith, G., 2005. Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. Vaccine 23, 5751–5759.
Quan, F.S., Vunnava, A., Compans, R.W., Kang, S.M., Virus-like particle vaccine protects against 2009 H1N1 pandemic influenza virus in mice. PLoS One 5, e9161.
Rao, S.S., Kong, W.P., Wei, C.J., Van Hoeven, N., Gorres, J.P., Nason, M., Andersen, H., Tumpey, T.M., Nabel, G.J., 2010. Comparative efficacy of hemagglutinin, nucleo-protein, and matrix 2 protein gene-based vaccination against H5N1 influenza in mouse and ferret. PLoS One 5, e9812.
Rebmann, T., Zelicoff, A., 2012. Vaccination against influenza: role and limitations in pandemic intervention plans. Expert Rev. Vaccines 11, 1009–1019.
Ross, T.M., Mahmood, K., Crevar, C.J., Schneider-Ohrum, K., Heaton, P.M., Bright, R.A., 2009. A trivalent virus-like particle vaccine elicits protective immune responses against seasonal influenza strains in mice and ferrets. PLoS One 4, e6032.
Schotsaert, M., De Filette, M., Fiers, W., Saelens, X., 2009. Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments. Expert Rev. Vaccines 8, 499–508.
Stephenson, I., Zambon, M.C., Rudin, A., Colegate, A., Podda, A., Bugarini, R., Del Giudice, G., Minutello, A., Bonnington, S., Holmgren, J., Mills, K.H., Nicholson, K. G., 2006. Phase I evaluation of intranasal trivalent inactivated influenza vaccine with nontoxigenic Escherichia coli enterotoxin and novel biovector as mucosal adjuvants, using adult volunteers. J. Virol. 80, 4962–4970.
Taubenberger, J.K., Baltimore, D., Doherty, P.C., Markel, H., Morens, D.M., Webster, R. G., Wilson, I.A., 2012. Reconstruction of the 1918 influenza virus: unexpected rewards from the past. mBio 3.
Treanor, J.J., Campbell, J.D., Zangwill, K.M., Rowe, T., Wolff, M., 2006. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N. Engl. J. Med. 354, 1343–1351.
Treanor, J.J., El Sahly, H., King, J., Graham, I., Izikson, R., Kohberger, R., Patriarca, P., Cox, M., 2011. Protective efficacy of a trivalent recombinant hemagglutinin protein vaccine (FluBlok(R)) against influenza in healthy adults: a randomized, placebo-controlled trial. Vaccine 29, 7733–7739.
Tumpey, T.M., Renshaw, M., Clements, J.D., Katz, J.M., 2001. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J. Virol. 75, 5141–5150.
Wan, H., Sorrell, E.M., Song, H., Hossain, M.J., Ramirez-Nieto, G., Monne, I., Stevens, J., Cattoli, G., Capua, I., Chen, L.M., Donis, R.O., Busch, J., Paulson, J.C., Brockwell, C., Webby, R., Blanco, J., Al-Natour, M.Q., Perez, D.R., 2008. Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One 3, e2923.
Wang, B.Z., Liu, W., Kang, S.M., Alam, M., Huang, C., Ye, L., Sun, Y., Li, Y., Kothe, D.L., Pushko, P., Dokland, T., Haynes, B.F., Smith, G., Hahn, B.H., Compans, R.W., 2007. Incorporation of high levels of chimeric human immunodeficiency virus envelope glycoproteins into virus-like particles. J. Virol. 81, 10869–10878.
Wang, T.T., Palese, P., 2009. Universal epitopes of influenza virus hemagglutinins? Nat. Struct. Mol. Biol. 16, 233–234.
Wei, C.J., Boyington, J.C., McTamney, P.M., Kong, W.P., Pearce, M.B., Xu, L., Andersen, H., Rao, S., Tumpey, T.M., Yang, Z.Y., Nabel, G.J., 2010. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. Science (New York, N.Y) 329, 1060–1064.
WHO, 2012a. Antigenic and genetic characteristics of influenza A(H5N1) and influenza A(H9N2) viruses and candidate vaccine viruses developed for potential use in human vaccines.
WHO, 2012b. WHO Manual on Animal Influenza Diagnosis and Surveillance, WHO/CDS/CSR/NCS/2002.5 Rev. 1.
WHO, 2013. Antigenic and genetic characteristics of A(H5N1), A(H7N3), A(H9N2) and variant influenza viruses and candidate vaccine viruses developed for potential use in human vaccines.
Yen, H.L., Webster, R.G., 2009. Pandemic influenza as a current threat. Curr. Top. Microbiol. Immunol. 333, 3–24.
Zeng, H., Goldsmith, C., Thawatsupha, P., Chittaganpitch, M., Waicharoen, S., Zaki, S., Tumpey, T.M., Katz, J.M., 2007. Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type i interferon response in polarized human bronchial epithelial cells. J. Virol. 81, 12439–12449.